

## Binding and Functional Properties of Recombinant and Endogenous CXCR3 Chemokine Receptors\*

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IP10 and MIG are two members of the CXC branch of the chemokine superfamily whose expression is dramatically up-regulated by interferon (IFN)- $\gamma$ . The proteins act largely on natural killer (NK)-cells and activated T-cells and have been implicated in mediating some of the effects of IFN- $\gamma$  and lipopolysaccharides (LPSs), as well as T-cell-dependent anti-tumor responses. Recently both chemokines have been shown to be functional agonists of the same G-protein-coupled receptor, CXCR3. We now report the pharmacological characterization of CXCR3 and find that, when heterologously expressed, CXCR3 binds IP10 and MIG with  $K_d$  values of 0.14 and 4.9 nM, respectively. The receptor has very modest affinity for SDF-1 $\alpha$  and little or no affinity for other CXC-chemokines. The properties of the endogenous receptor expressed on activated T-cells are similar. Surprisingly, several CC-chemokines, particularly eotaxin and MCP-4, also compete with moderate affinity for the binding of IP10 to CXCR3. Eotaxin does not activate CXCR3 but, in CXCR3-transfected cells, can block IP10-mediated receptor activation. Eotaxin, therefore, may be a natural CXCR3 antagonist.

Chemokines are a superfamily of small secreted proteins that play an important role in the selective trafficking of leukocytes (for review, see Ref. 1). Most members of the superfamily can be divided into two groups depending on the organization of the first cysteine pair: the CC branch in which the cysteines are adjacent, and the CXC-branch in which they are separated by a single amino acid. Two members of the CXC branch of the superfamily, IP10 and MIG, were initially identified because of their dramatically enhanced expression in monocytes activated by IFN- $\gamma$ <sup>1</sup> or LPS (2–4). The biological actions of IP10 and MIG are largely restricted to activated T- and natural killer (NK)-cells for which both are potent chemoattractants (5, 6). These properties suggest that the two chemokines may mediate some of the lymphocyte-directed effects of IFN- $\gamma$  and LPS, a hypothesis that is supported by the observation that IP10 elicits a potent T-cell-dependent antitumor response (5). IP10 and MIG are also strongly angiostatic

(7), a property which may be related to their antitumor activity.

Chemokines elicit their biological functions by binding to specific G protein-coupled receptors expressed on the appropriate cells types. Like the chemokines, the receptors can be largely divided into two sub-families: the CXC receptors (CXCRs), which bind CXC chemokines, and the CC receptors (CCRs), which bind CC-chemokines (1). Recently, Loetscher *et al.* (8) identified a new member of the CXCR subfamily, CXCR3, which when recombinantly expressed mediated chemotaxis and  $Ca^{2+}$  mobilization in response to both IP10 and MIG. However, these authors were unable to show binding of either ligand to CXCR3. We now demonstrate that CXCR3 does bind both IP10 and MIG with affinities consistent with the concentrations of the chemokines required to elicit cellular responses. In addition, CXCR3 has some avidity for the CXCR4 ligand, SDF-1, and rather surprisingly has considerable affinity for several CC-chemokines, particularly the CCR3 ligands eotaxin and MCP-4. The pharmacological properties of the recombinantly expressed receptor mirror those of the native receptor expressed on activated human T-cells.

### EXPERIMENTAL PROCEDURES

**Materials**—All human chemokines were from Peptrotech (Rocky Hill, NJ) except for SDF-1 $\alpha$ , which was from Gryphon Sciences (South San Francisco, CA). Radioactive chemokines were from NEN Life Science Products. Venous whole blood or plasmapheresed leukocytes from normal human donors was obtained from the New York Blood Center or the University of Pennsylvania Medical Center.

**Cloning of CXCR3**—The cDNA encoding CXCR3 was cloned by PCR using lymph node cDNA (CLONTECH, Palo Alto, CA) as a template. The PCR primers were designed based on the published sequence (8). The PCR product was digested with *EcoRI* and *NotI* and ligated to similarly digested and linearized pBluscript KS II\* (Promega). The sequence of the coding region of the receptor was verified by sequencing, then excised from pBS-CXCR3 by digestion with *HindIII* and *NotI*, and then ligated into mammalian expression vector pBJ-neo (9).

**Expression of CXCR3 in CHO and RBL-2H3 Cells**— $10^6$  CHO cells (ATCC: CCL-61) were transfected with 20  $\mu$ g of DNA using a standard calcium phosphate procedure (Specialty Media, Lavallette, NJ). The DNA was incubated with cells at 37 °C, 6%  $CO_2$  for 6 h, whereupon the cells were glycerol-shocked (15% glycerol shock solution, Specialty Media) and re-fed with selection media containing 0.4 mg/ml Geneticin (Life Technologies, Inc.). Concurrently, RBL-2H3 cells were electroporated with the CXCR3 expression plasmid as described previously (13) and selected in 1 mg/ml Geneticin. After 10 days, the surviving CHO or RBL foci were pooled. Stable expression of CXCR3 was verified by determining that an aliquot of cells bound radiolabeled IP10 (see binding parameters below). The remaining cells were cloned by limiting dilution in 96-well microtiter plates, and the cells were expanded. Stable cell lines were derived from individual clones selected on the basis of binding and functional assays.

**Binding Assays**—Binding of  $^{125}I$ -IP10 (2200 Ci/mmol, typically 20 pM) in the presence of unlabeled ligands was initiated by adding intact cells (75,000 cells/point) as described previously (10). After incubation at room temperature for 30 min, the cells were filtered through GF/C filters treated with 0.33% polyethyleneimine and washed with buffer

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<sup>1</sup> The abbreviations used are: IFN, interferon; LPS, lipopolysaccharide; CXCR, CXC receptor; CCR, CC receptor; PCR, polymerase chain reaction; CHO, Chinese hamster ovary.

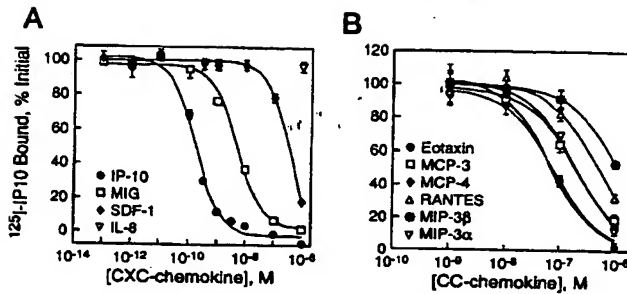


Fig. 1. Pharmacology of CXCR3 recombinantly expressed in CHO cells. Increasing concentrations of unlabeled chemokines were used to compete against a fixed concentration of  $^{125}\text{I}$ -IP10. Shown are the results obtained using CXC chemokines as competitors (A) and the results with CC-chemokines (B). Data are from single representative experiments. No binding of  $^{125}\text{I}$ -IP10 was observed in nontransfected or mock-transfected CHO control cells.

containing 25 mM HEPES, 0.02% NaN<sub>3</sub>, and 0.5 M NaCl, pH 7.2.

**Microphysiometry**—Functional assays were performed using microphysiometry (11, 12). Briefly, untransfected CHO-K1 cells or CHO-K1 cells stably transfected with human chemokine receptor CXCR3 were seeded onto the Transwell cell capsule cups (Molecular Devices, Sunnyvale, CA) at a density of  $0.33 \times 10^6$  cells/ml/cup in Ham's F-12 medium plus 10% fetal bovine serum. Following overnight culture, the capsules were transferred to microphysiometer sensor chambers (Cytosensor, Molecular Devices) and allowed to equilibrate for 2 h, during which time they were perfused with running medium (1 mM phosphate-buffered RPMI 1640 medium, pH 7.4 (Molecular Devices) plus 0.1% bovine serum albumin (Life Technologies, Inc.)). Once stable acidification rates were established, cells were exposed for 6 min to various concentrations of chemokine diluted in running medium. A flow rate of 100  $\mu\text{l}/\text{min}$  was used, and acidification rates were measured at 2 min intervals.

**Calcium Flux**—Measurements were carried out with transfected cells or purified T-cells labeled with Indo-1 (Molecular Probes, Eugene, OR) as described previously (13).

**T-cell Activation**—T-cells were purified from a mononuclear cell preparation by E-rosetting with neuraminidase-treated sheep red blood cells, followed by overnight incubation at 37 °C. The T-cells were washed and incubated in plastic flasks at  $2 \times 10^6/\text{ml}$  in media containing 400 units/ml of human recombinant IL-2 (Biosource International, Camarillo, CA) for 1–2 days, and further maintained at a density of  $2\text{--}4 \times 10^6/\text{ml}$  in fresh media containing IL-2 at 200 units/ml for the specified times.

## RESULTS AND DISCUSSION

**Characterization of the Binding Properties of CXCR3 on CHO Cells Stably Expressing the Recombinant Receptor**—Because untransfected CHO cells do not bind  $^{125}\text{I}$ -IP10, lines stably expressing CXCR3 were established by monitoring the gain of this activity (see "Experimental Procedures"). The binding properties of a representative CHO clone (C1.17) were assessed by competition of various chemokines against  $^{125}\text{I}$ -IP10 (Fig. 1 and Table I). The clone exhibits a single high affinity binding site ( $K_i = 0.14$  nM) for IP10 and expresses approximately 50,000 sites/cell. As expected, the receptor also binds MIG, although with an affinity ( $K_i = 4.9$  nM) that is substantially lower than that found for IP10. Other CXC-chemokines, notably IL-8, which binds to both CXCR1 and CXCR2 (14, 15), and GRO $\alpha$  and NAP2, which bind to CXCR2, have little or no affinity for CXCR3 (Table I). SDF-1, the ligand for CXCR4 (16, 17), does show very slight affinity for CXCR3 with a  $K_i = 400$  nM.

Rather surprisingly, a number of CC-chemokines show moderate affinity for CXCR3, in particular the ligands for CCR3 (9, 18). Eotaxin and MCP-4 have  $K_i$  values of 60 and 70 nM, whereas MCP-3 and RANTES have lower affinities with  $K_i$  values of 250 and 420 nM, respectively. Because CCR6 and CCR7 are phylogenetically more closely related to CXCR3 than is CCR3, we also examined the ability of their ligands (MIP-3 $\alpha$

TABLE I  
Affinities of CXC- and CC-chemokines for CXCR3

$K_i$  values were determined from competition binding experiments carried out against  $^{125}\text{I}$ -IP10 as described in the legends of Figures 1 and 2 and in the "Experimental Procedures" section. All values are given in nM and are the averages of two to three experiments. NB is no binding; ND not determined.

Ligand	$K_i$ , nM		
	CHO line	Activated T-cells	RBL line
IP10	$0.14 \pm .01$	$0.04 \pm .01$	$0.47 \pm 0.16$
MIG	$4.9 \pm .3$	$0.8 \pm .2$	$3.3 \pm 1.3$
SDF-1 $\alpha$	$400 \pm 300$	$170 \pm 20$	ND
GRO $\alpha$	>1000	NB	ND
IL-8	NB	NB	ND
NAP2	NB	ND	ND
PF4	NB	ND	ND
Eotaxin	$60 \pm 8$	$70 \pm 30$	$58.5 \pm 6.5$
MCP-4	$70 \pm 2$	$40 \pm 10$	ND
MCP-3	$250 \pm 100$	$100 \pm 20$	ND
RANTES	$420 \pm 20$	$130 \pm 30$	ND
MIP-3 $\alpha$	$140 \pm 20$	ND	ND
MIP-3 $\beta$	$1300 \pm 400$	ND	ND

and MIP-3 $\beta$ ) to bind to CXCR3. MIP-3 $\alpha$  does bind with some affinity ( $K_i = 160$  nM), although MIP-3 $\beta$  shows very little activity ( $K_i = 1700$  nM). Similarly, MIP 1 $\alpha$ , MIP1 $\beta$ , and MCP-1, ligands for CCRs 1, 2, and 5 (reviewed in Ref. 1) show little or no affinity for the receptor (data not shown).

**Binding of IP10 and Other CXCR3 Ligands to Purified IL-2 Activated T-cells**—Since a number of factors, including differences in the endogenous complement of G-proteins or various other host cell-specific restrictions, may influence the way in which ligands bind to their receptors, we compared the pharmacology of the receptor recombinantly expressed in CHO cells to endogenously expressed CXCR3 and to receptor expressed in RBL-2H3 cells.

It has been reported that transcripts for CXCR3 are virtually absent in resting T-cells but are present in IL-2-activated T-cells (8). In our hands, the ability of human T-cells to bind IP10 is consistent with these observations. Freshly isolated T-cells show variable but low binding activity, an activity that is substantially up-regulated by a number of activation procedures including treatment with anti-CD3/anti-CD28, phorbol 12-myristate 13-acetate/ionomycin, or IL-2 (data not shown). To characterize the pharmacology of CXCR3 on primary cells, we chose to use T-cells treated with IL-2 for 6–8 days, a protocol that generates maximal binding of IP10 (data not shown). As shown in Fig. 2 and Table I, the properties of the receptor on the primary cells closely mirrors that of the recombinant molecule expressed in CHO cells. The  $K_i$  cells are 2–3-fold lower on average, but the receptors in both cell types bind the same ligands with the same rank order of potencies. Similar affinities were also obtained for receptor recombinantly expressed in RBL-2H3 cells (Table I).

**Functional Responses Induced by Chemokines through CXCR3**—The earlier identification and characterization of CXCR3 was carried out by assessing the ability of various CXC- and CC-chemokines to mobilize calcium in murine 300–19 pre-B cells transfected with the receptor. In these experiments, Loetscher *et al.* found IP10 and MIG to be the only chemokines to induce a response, and as a consequence, they referred to CXCR3 as the IP10/MIG receptor. To confirm and extend this preliminary characterization and to determine whether the concentrations of IP10 and MIG required for functional activation reflect the ligands' differential affinities, we also evaluated the functional properties of the receptor in the stably transfected CHO cell line. Since in our hands CHO cells are refractory to the uptake of calcium sensitive dyes, a microphysiom-

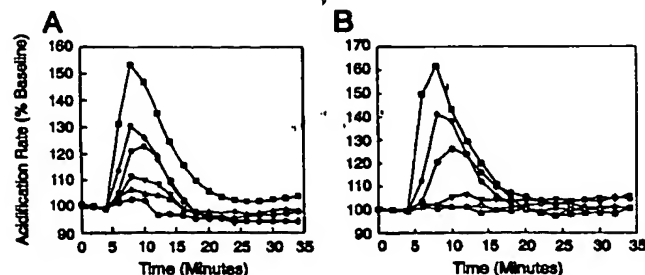


FIG. 2. Microphysiometric analysis of change in acidification rate of CXCR3 expressing CHO cells in response to addition of IP10 (panel A: 100 nM (■), 30 nM (◇), 10 nM (●), 3 nM (▽), 1 nM (Δ), or 100 pM (+)), MIG (panel B: 1 μM (■), 300 nM (◇), 100 nM (+), 30 nM (▽), 10 nM (▼)) or eotaxin (panel B: 1 μM (○)). Cellular responses were monitored at 2-min intervals following addition of chemokine. No response was induced in untransfected CHO cells by IP10 or MIG at concentrations up to 1 μM. Results with MCP-4 and SDF-1α are not shown because these chemokines generated a response at 1 μM in untransfected cells.

eter was used to monitor ligand-induced increases in cellular acidification rate as a measure of functional activation (11).

As illustrated in Fig. 3 both IP10 and MIG induce dose-dependent increases in the acidification rate. As expected from the binding data, IP10 is more potent than MIG with  $EC_{50}$  values of ~10 and 100–200 nM, respectively. In fact, the ratio of  $EC_{50}$  values for the two ligands (10–20) is consistent with the relative binding affinities (~30, Table I). In comparison, eotaxin failed to generate any response, even at a concentration of 1 μM (Fig. 3B), a value 15-fold greater than its  $K_i$  as determined in binding studies (Table I).

We also examined the ability of IP10, MIG, and eotaxin to stimulate functional responses in activated human T-cells and transfected RBL cells. For these studies, increases in intracellular  $Ca^{2+}$  levels were used to monitor responses. The data are consistent with the results from the recombinant CHO lines. As shown in Fig. 4, in activated primary T-cells, both IP10 and MIG induced a flux at a concentration of 100 nM, whereas eotaxin failed to generate a response, even at a concentration of 1 μM. Interestingly, since a subset of Th2 T-cells has been reported to express CCR3 (19), the primary receptor for eotaxin, the lack of response to eotaxin also suggests that our T-cell preparations contain a very low level of this subset. High concentrations of eotaxin (up to 1 μM) also failed to induce a  $Ca^{2+}$ -flux in our stable RBL CXCR3 transfected line (Fig. 5), whereas the  $EC_{50}$  of IP10 for this response was 10 nM (data not shown).

The binding and functional data suggest that eotaxin, under appropriate circumstances, should act as a receptor antagonist. To test this hypothesis, we examined the ability of eotaxin to inhibit an IP10-induced  $Ca^{2+}$ -flux in the transfected RBL cells. As shown in Fig. 5, eotaxin does inhibit the response to 10 nM IP10 with an  $IC_{50}$  of about 1 μM, a potency consistent with the 100-fold difference in binding affinities (Table I). As a control, IL-8 had no effect at concentrations as high as 10 μM.

The chemokine system consists of more than 50 ligands and 13 receptors. The specificities of the ligand/receptor interactions are complex as each receptor binds multiple chemokines and most chemokines bind to more than one receptor. However, a general rule has been that a chemokine receptor binds either CC- or CXC-chemokines but not both. The one exception has been DARC, a highly promiscuous, ubiquitously expressed, non-signaling receptor whose function is unclear (20, 21). Thus it is surprising to find that CXCR3 has moderate affinity for several CC-chemokines, particularly those which bind to CCR3. In fact, those affinities are higher than for any of the

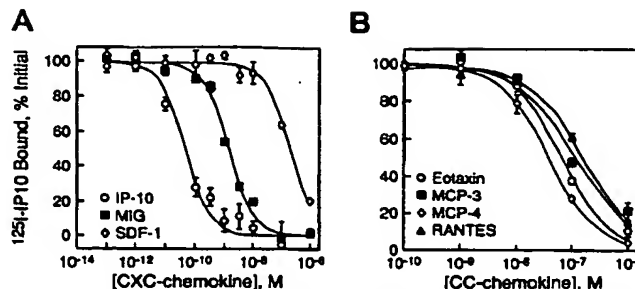


FIG. 3. Pharmacology of the IP10 receptor (CXCR3) expressed on activated T-cells. Cells were activated for 6–8 days with recombinant human IL-2, and the binding properties of CXCR3 were determined as described in Fig. 1, except that the assays employed 250,000 cells/point. Panel A shows the results obtained using CXC chemokines as competitors, and panel B shows the results with CC-chemokines. Data are from single representative experiments.

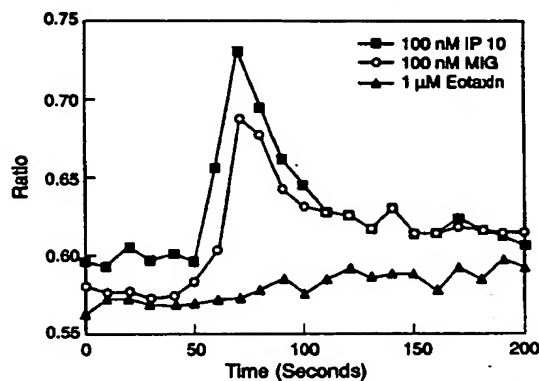


FIG. 4. IP10 and MIG, but not eotaxin, induce mobilization of intracellular  $Ca^{2+}$  in CXCR3 expressing IL-2-activated human T-cells. Cells were cultured as described in the legend for Fig. 4 prior to analysis.

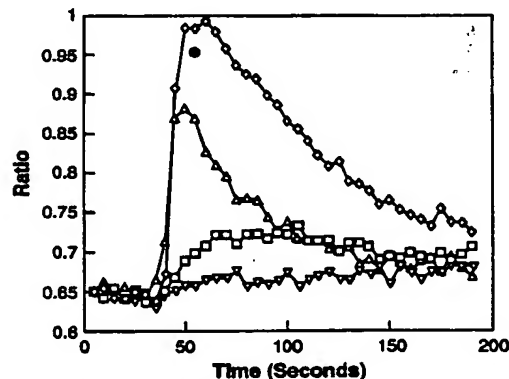


FIG. 5. Eotaxin inhibits IP10-mediated CXCR3-dependent  $Ca^{2+}$  mobilization in RBL CXCR3 cells. The data represent  $Ca^{2+}$  fluxes mediated by 10 nM IP10 in cells pretreated 5 min before analysis with buffer alone (○) or a final concentration of either 1 μM (Δ) or 3 μM (□) eotaxin. No further suppression of the IP10 response was achieved by extending the titration of eotaxin above 3 μM. A single point reflecting the maximal functional activation of the receptor to IP10 after pretreatment with 10 μM IL-8 (●) shows that the effect of eotaxin is specific since the slight diminution in response is within the limits of experimental variability. Eotaxin at a concentration of 3 μM (▽) does not itself induce a flux in these cells.

CXC-chemokines examined here, except IP10 and MIG. It would appear, therefore, that overall protein sequence homologies are not a sufficient means from which to predict the class

of ligand (CC or CXC) a given receptor may bind. Moreover, since the primary CXCR3 ligands, IP10 and MIG, also show moderate affinities for CCR3 ( $K_i$  values of 100 and 30 nM, respectively<sup>2</sup>), it is also likely that CXCR3 and CCR3 possess structural homology that enables them to present key interactions to shared ligands.

An important but as yet unanswered question, given the modest affinities that the CCR3 ligands show for CXCR3 and vice versa, is whether these overlapping ligand specificities are physiologically relevant. It is tempting to believe they are. *In vivo*, there is a correlation between strong TH2 responses and the diminished accumulation of TH1 cells (22, 23). Since CCR3 is expressed on cells characteristic of TH2 responses, including a subset of TH2 T-cells (19, 24) and whereas CXCR3 is found predominately on TH1 T-cells (24), the putative antagonistic effects of the CCR3 ligands on CXCR3 could impair the accumulation of TH1 cells and in part account for some of the inhibitory activity of TH2 responses. Considerable additional evidence is needed to support this speculation, including the direct demonstration that CCR3 ligands do indeed antagonize the effects of IP10 and MIG on TH1 cells. Moreover, sufficient local concentrations of the CCR3 chemokines have to be achieved in order for such antagonism to occur. In this regard, it is currently thought that much of the chemokine generated *in vivo* is bound to surface proteoglycan, a modality that has been argued to greatly increase the local concentration, particularly because the interaction with the receptor occurs on a solid phase (25).

Regardless of whether the above argument is true, our data suggest that chemokines may play a dual regulatory role, as agonists for some responses and antagonists for others.

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<sup>2</sup> Y. Weng, S. J. Siciliano, K. E. Waldburger, A. Sirotina-Meisher, M. J. Staruch, B. L. Daugherty, S. L. Gould, Martin S. Springer, and Julie A. DeMartino, unpublished observations.

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